



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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| <b>(51) International Patent Classification <sup>6</sup> :</b><br><b>C07K 14/78, A61K 47/42, G01N 33/543</b>  | <b>A1</b> | <b>(11) International Publication Number:</b> <b>WO 96/41817</b><br><b>(43) International Publication Date:</b> 27 December 1996 (27.12.96)  |
| <b>(21) International Application Number:</b> PCT/EP96/02453<br><b>(22) International Filing Date:</b> 6 June 1996 (06.06.96)<br><b>(30) Priority Data:</b><br>95108967.1 10 June 1995 (10.06.95) EP<br><i>(34) Countries for which the regional or international application was filed:</i> CH et al.<br><b>(71) Applicant (for all designated States except US):</b> PEN-TAPHARM AG [CH/CH]; Engulgasse 109, CH-4052 Basel (CH).<br><b>(72) Inventors; and</b><br><b>(75) Inventors/Applicants (for US only):</b> STOCKER, Kurt [CH/CH]; Blumenrain 12, CH-4147 Aesch (CH). GAFFNEY, Patrick, J. [IE/GB]; 27 Milton Road, Harpen-ten, Herts AL5 5LA (GB).<br><b>(74) Agent:</b> BRAUN, André; Murtengasse 5, CH-4051 Basel (CH).  |           | <b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).<br><br><b>Published</b><br><i>With international search report.</i><br><i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i> |
| <b>(54) Title:</b> COLLAGEN PEPTIDE FRACTION AND ITS USES<br><br><b>(57) Abstract</b><br><br><p>It was found that a collagen peptide fraction (CPF) of especially porcine collagen &gt;70 %, especially &gt;80 %, of which have an average molecular weight of 8-30 kDa, a hydroxyproline content of 15-19 %, a proline content of 18-22 % and a glycine content of 27-33 % which is stable at temperatures up to 150 °C, and shows a viscosity of 2.0 to 3.0 cSt [mm<sup>2</sup>/sec] at a concentration of 10 % in water and an UV absorbance at 280 nm of 0.6 to 1.8 has a surprising stabilising effect on protein and polypeptides in freeze dried form and in solution. CPF-stabilised proteins and polypeptide drugs can be used during long term infusion or for topical, nasal or transdermal application.</p> |           |  |

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COLLAGEN PEPTIDE FRACTION AND ITS USES

The present invention relates to a new collagen peptide fraction (CPF) consisting of several peptide species which can among others be used for the stabilisation of protein and peptide drugs during long term infusion, of liquid forms of protein and peptide drugs and of freeze dried respectively lyophilised proteins and peptides or freeze dried respectively lyophilised drugs containing proteins and peptides for topical, nasal or transdermal application.

Biologically active proteins in their natural environment are stabilized within a balanced system of biopolymers, carbohydrates and electrolytes. Maintenance of stability during handling and storage of highly purified proteins and polypeptides for therapeutic and other uses requires special procedures.

Polypeptide and protein drugs are currently stabilized by lyophilization and have to be reconstituted before application. Lyophilized pharmaceutical preparations in general may contain bulking agents (eg amino acids, carbohydrates or polyalcohols), inorganic or organic buffer substances, electrolytes and bacteriostatics. Every single component of a freeze dried preparation has to be compatible with each other and the total composition has to provide an optimum environment for the stability of the active ingredient in the freeze dried state.

A composition that provides stability in a freeze dried state is not necessarily best for drug stability in solution after reconstitution. This is of minor importance if the preparation is injected immediately after reconstitution. It may cause major problems, however, if the reconstituted drug has to be continuously infused during a prolonged time period. For this purpose the

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composition of the respective pharmaceutical preparation has to guarantee maximum stability in the freeze dried state as well as after reconstitution.

Maintenance of protein and polypeptide drug stability in solution may also be a major problem in topical, nasal or transdermal drug delivery systems including entrapped or encapsulated preparations of drug solutions.

Loss of protein activity in solution may be caused by sub-optimal pH and ionic strength, autocatalytic degradation, heat, oxygen, surface denaturation, adsorbant surfaces, shear forces, high pressure, irradiation, etc. For a review on protein inactivation see M.P. Deutscher, Maintaining protein stability. In: Guide to protein purification, M.P. Deutscher (Ed), Meth. in Enzymol. Vol. 182, 83-89, Academic Press: San Diego, New York, Berkeley (1990).

Activity loss of proteins and polypeptides in solution due to physical actions can best be prevented by the addition of inert proteins in excess. Thus, human serum albumin (HSA), bovine serum albumin (BSA) and ovalbumin are frequently used as protein stabilizing agents. However, BSA and ovalbumin are strong antigens and can therefore not be used for the stabilization of injectable drugs. Human serum albumin is not antigenic to humans but it can bear a risk of viral (HIV, hepatitis) and mycoplasma contamination. A further disadvantage of HSA as a stabilizing agent involves the possible contamination with other biologically active materials eg proteinases or proteinase inhibitors that may interact with the protein to be stabilized (see M.C.E. Van Dam-Mieras, A.D. Muller, H.C. Hemker, Blood coagulation factors II, V, VII, VIII, IX, X and XI: Determination by clotting assays. In: Methods of enzymatic analysis, 3rd edition, Vol. 5, Enzymes 3, p.352-365, H.U. Bergmeyer (Ed), Verlag Chemie: Weinheim, Deerfield Beach, Basel (1984) and D.B. Gubler, B.D. Wilson, C.J. Parker, G.M. Rodgers,

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Regulation of endothelial cell protein C activation and fibrinolysis by procoagulant albumin. *Thromb. Res.* 70, 459-469 (1993). HSA is similarly disadvantageous when used in topical applications.

It has been discovered that collagen from pig skin, a virtually non-antigenic major constituent of animal tissues, after solubilization with pepsin, exerts a stabilizing effect on thrombin in solution as well as in a freeze dried state. However, solubilized collagen cannot be used in injectable preparations because of its activating effect on platelet aggregation. A further disadvantage of solubilized collagen is its high viscosity in solution and its instability. Heating over 40°C causes denaturation of collagen and subsequent gel formation upon cooling to room temperature. Addition of phosphate anions to collagen causes the formation of an insoluble gel.

However, it was found that collagen, following partial acid hydrolysis into lower molecular peptides, could still exhibit protein stabilising properties, did not cause platelets to aggregate, formed low viscosity solutions and was compatible with current buffering substances including phosphates. This was named as a collagen peptide fraction (CPF).

It was also found that solutions of biologically active protein drugs which are highly susceptible to physico-chemical inactivation (eg thrombin, tissue plasminogen activator, urokinase, antithrombin and other plasma proteins) were more stable in CPF prepared from porcine skin collagen than in solutions which contained human serum albumin.

It was finally found that CPF was capable of saturating or blocking protein adsorbing or covalently binding sites of glass and synthetic or natural polymers in preparative,

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analytical, diagnostic and medical devices such as microtiter plates, blotting membranes, filters, tubings etc. A treatment by rinsing or incubation with CPF solution can therefore be used to prevent unspecific antibody or antigen binding in immunological techniques such as enzyme linked immuno adsorption (ELISA), immunoblotting and related procedures. Incubation with CPF can also be used to saturate excessive active groups in activated supports for affinity chromatography, and washing with CPF of filter material, glass or plastic ware will prevent adsorption of proteins from solutions during processing.

It is therefore an object of the invention to use collagen peptide fraction (CPF) for the stabilisation of protein and peptide drugs during long term infusion.

It is another object of the invention, to use collagen peptide fraction (CPF) for the stabilisation of liquid forms of protein and peptide drugs for topical, nasal or transdermal application.

It is still another object of the invention, to use collagen peptide fraction (CPF) for the stabilisation of freeze dried proteins and peptides or freeze dried drugs containing proteins and peptides for topical, nasal or transdermal application.

It is still another object of the invention, to use collagen peptide fraction (CPF) to prevent unspecific antibody or antigen binding in immunological techniques such as enzyme linked immuno adsorption (ELISA), immunoblotting and related procedures.

It is still another object of the invention, to use collagen peptide fraction (CPF) to saturate excessive active groups in activated supports for affinity chromatography.

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It is still another object of the invention, to use collagen peptide fraction (CPF) to wash filter material, glass or plastic ware to prevent adsorption of proteins from solutions during processing.

Collagen suitable for the production of a collagen peptide fraction (CPF) according to the present invention is obtainable from animal tissues, preferably pig skin. Pepsin solubilized type I collagen can be prepared by conventional techniques eg according to the method of N D Light, 1985. Collagen in Skin: Preparation and Analysis, in: Methods in Skin Research (D Skerrow and C J Skerrow, Eds) J Wiley and Sons Ltd. p 559-585.

Controlled hydrolysis of type I collagen can be performed by heating an aqueous collagen suspension at low pH for a defined time period. A collagen peptide fraction (CPF) according to the invention is obtainable by heating a collagen suspension at pH 2.5-4.0 for 30-90 minutes at 100-150°C in an autoclave. The parameters (pH, temperature, time) can be varied to a wide extent. E.g. low pH and high temperature will reduce the heating time.

A collagen peptide fraction (CPF) according to the present invention consists of several peptide species, >70%, especially >80%, of which have an average molecular weight of 8-30 kDa, especially 8 to 25 kDa, more especially 10-20 kDa, most especially 20 kDa (estimated by gel permeation chromatography), a hydroxyproline content of 15-19%, a proline content of 18-22% and a glycine content of 27-33% (according to D.H. Spackman et al., Anal. Chem. 30, 1190-1206, (1958)). In contrast to serum and egg albumins which precipitate from aqueous solution upon heating at >60°C, CPF is stable and does not precipitate at temperatures up to 150°C and may thus be sterilized by heating in an autoclave. As opposed to collagen which at a concentration of 10% in water is a sticky paste and to 10%

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gelatin which at room temperature is a solid gel, CPF in 10% aqueous solution remains fluid and shows a viscosity of 2.0 to 3.0 cSt [mm<sup>2</sup>/sec]. Unlike albumins which show strong UV absorbance at 280 nm ( $A_{280\%} = 6.2$  to 7.5), CPF shows  $A_{280\%}$  values of 0.6 to 1.8 (1% in water, corresponding to 0.1% solids), depending on the mode of preparation. A mixture of equal volumes of 10% aqueous CPF solution and 10% trichloro-acetic acid does not form any protein precipitate while albumin or gelatin, under similar conditions form strong precipitates. A CPF solution in 5% ammonia, after heating to 95°C with 1 ml silver nitrate, 0.1 M, does not show any brownish colouration, whereas gelatin under similar conditions, due to its content of reducing carbohydrates, shows a dark brown colour. In gelatin, reducing carbohydrates originating from glucosaminoglycan degradation, produce a strong orcinol colour reaction whereas CPF shows a very weak reaction only.

#### Example 1 Preparation of the collagen peptide fraction (CPF)

Type I collagen from pig skin was purified according to Light (see ref. above). Freshly frozen pig skin was ground and defatted by solvent extraction. The resulting skin fibre pulp was treated with pepsin to solubilize type I collagen. Insoluble material was removed by filtration, collagen was precipitated from the filtrate at pH 7.5, dissolved in saline and further purified by salt fractionation and ion exchange treatments. Precipitated type I collagen was suspended in water, the pH adjusted to 3.5 with hydrochloric acid, the acidified suspension was heated in an autoclave for 60 min at 145°C, the concentration was adjusted with water to  $10 \pm 1\%$  solids. This solution was filtered under aseptic conditions through a 0.2  $\mu$ m membrane to yield a sterile, 10% solution of collagen peptides with an endotoxin content below 10 endotoxin units per ml and a heavy metal



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content below 20 ppm. As estimated by analytical gel chromatography, the average molecular weight of >80% of the obtained collagen peptide fraction (CPF) is 10-15 kDa.

|                          |             |
|--------------------------|-------------|
| Average molecular weight | 10 - 15 kDa |
| Total nitrogen           | 15 - 19%    |
| Polypeptide content      | 80 - 100%   |
| Hydroxyproline           | 15 - 19%    |
| Proline                  | 18 - 22%    |

#### Example 2

Human thrombin (=TH, a procoagulant enzyme widely used as a topical haemostat and as a component of tissue sealants) and tissue plasminogen activator (=TPA, a popular thrombolytic agent used for the treatment of various forms of acute and chronic thrombosis) were incubated in the presence of HSA (0.5% solution) and collagen peptide fraction (CPF, 0.5% solution) for seven days at various temperatures (the various storage temperatures are shown on the abscisse, =temp). Figure 1 shows that the biological activity of thrombin (shown as residual activity ratio, =rar) was significantly maintained in the presence of CPF compared with HSA. Figure 2 shows that the activity of TPA which is commonly used in therapy as an infusion also suffered less loss of activity when incubated with CPF rather than HSA at 37°C.

#### Example 3

Tissue plasminogen activator (=TPA), thrombin (=TH) and alkaline phosphatase (=AP, a highly susceptible enzyme to degradation during preservation by lyophilisation) were lyophilized from solutions of CPF and HSA and stored at a variety of temperatures (=temp) for 12 weeks (Figures 3, 4 and 5). The loss in the activities during lyophilisation was approximately similar for CPF and HSA and the potencies of

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the enzymes lyophilized from CPF solutions were similar to those lyophilized from HSA solutions following storage for 12 weeks over a wide range of temperatures. Only at a severely elevated temperature (e.g. 56°C) did the lyophilized CPF product show an advantage in the maintenance of potency over the lyophilized HSA product (especially Figures 3 and 4). These latter data at 56°C suggest strongly that long term storage (> 1 year) of lyophilized biological activities may be enhanced by the use of the CPF fraction.

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Claims

1. Collagen peptide fraction (CPF) consisting of several peptide species >70%, especially >80%, of which have an average molecular weight of 8-30 kDa, especially 8-25 kDa, more especially 10-20 kDa, more especially 20 kDa, a hydroxyproline content of 15-19%, a proline content of 18-22% and a glycine content of 27-33% which is stable at temperatures up to 150°C, and shows a viscosity of 2.0 to 3.0 cSt [mm<sup>2</sup>/sec] at a concentration of 10% in water and UV absorbance at 280 nm of 0.6 to 1.8 at a concentration of 1% in water.
2. Method for the preparation of a collagen peptide fraction (CPF) according to claim 1, characterized by heating a collagen suspension at pH 2.5-4.0 for 30-90 minutes at 100-150°C in an autoclave.
3. Method according to claim 2, characterized by a pH adjusted to 3.5 preferably with hydrochloric acid and heating the fraction for 60 min at 145°C, preferably in an autoclave.
4. Method according to claim 2 or 3, using as starting material essentially porcine collagen.
5. Use of a collagen peptide fraction (CPF) according to one of claims 1-4 for the stabilisation of protein and peptide drugs during long term infusion.
6. Use of a collagen peptide fraction (CPF) according to one of claims 1-4 for the stabilisation of liquid forms of protein and peptide drugs for topical, nasal or transdermal application.
7. Use of a collagen peptide fraction (CPF) according to one of claims 1-4 for the stabilisation of freeze dried respectively lyophilised proteins and peptides or freeze

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dried respectively lyophilised drugs containing proteins and peptides for topical, nasal or transdermal application.

8. Use of a collagen peptide fraction (CPF) according to one of claims 1-4 to prevent unspecific antibody or antigen binding in immunological techniques such as enzyme linked immuno adsorption (ELISA), immunoblotting and related procedures.

9. Use of a collagen peptide fraction (CPF) according to one of claims 1-4 to saturate excessive active groups in activated supports for affinity chromatography,

10. Use of a collagen peptide fraction (CPF) according to one of claims 1-4 to prevent adsorption of proteins from solutions during processing.

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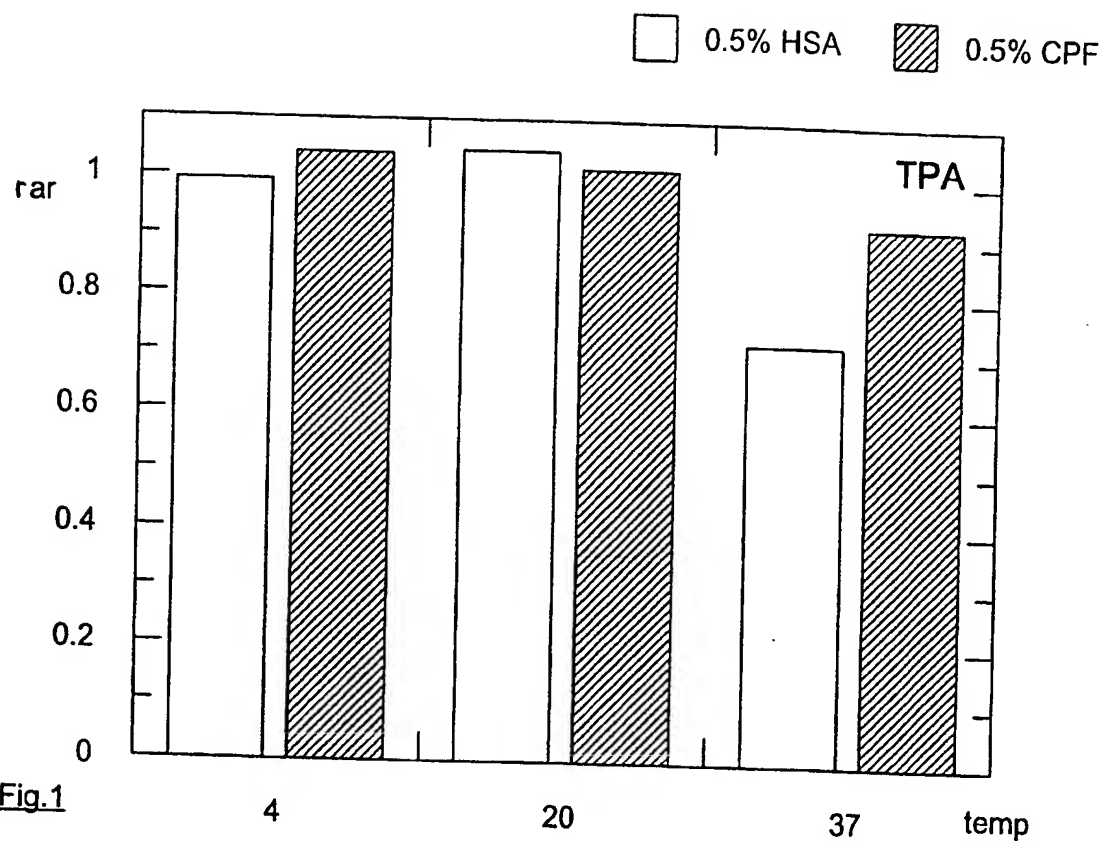


Fig.1

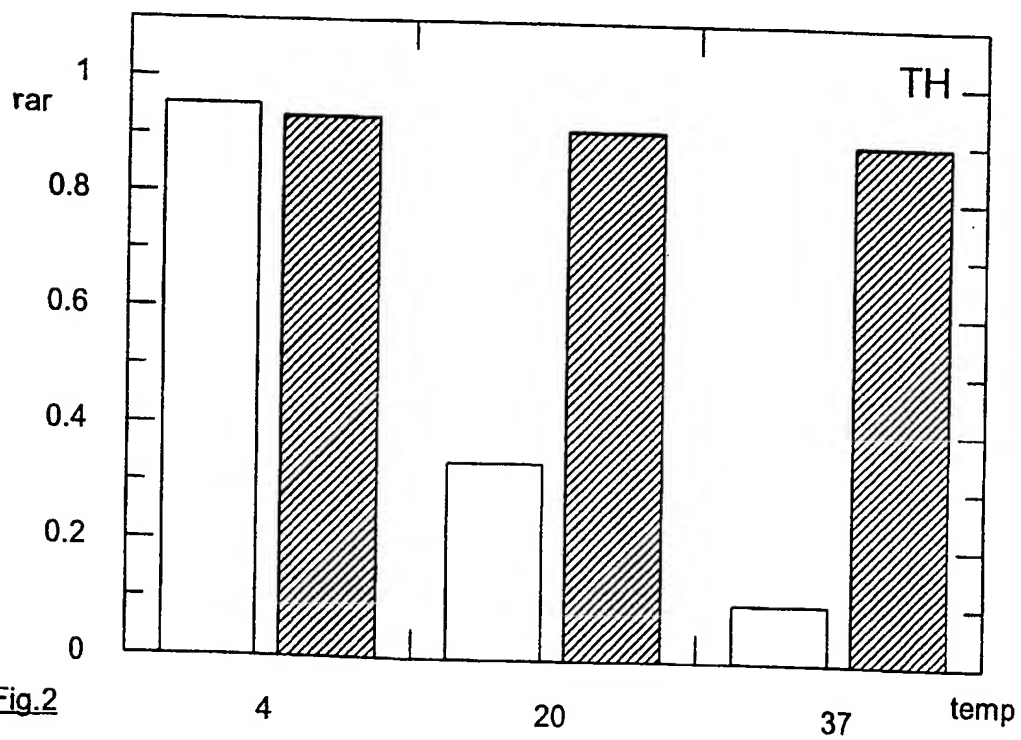
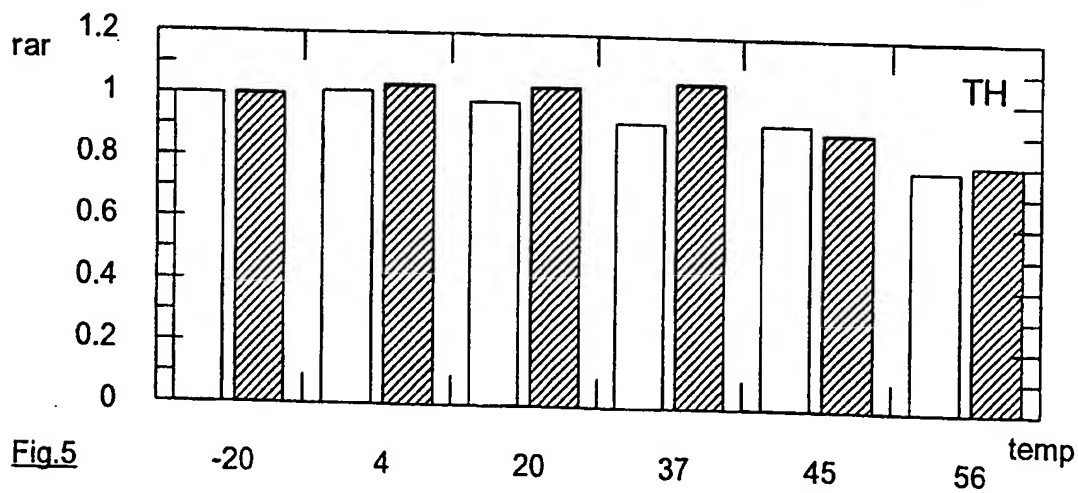
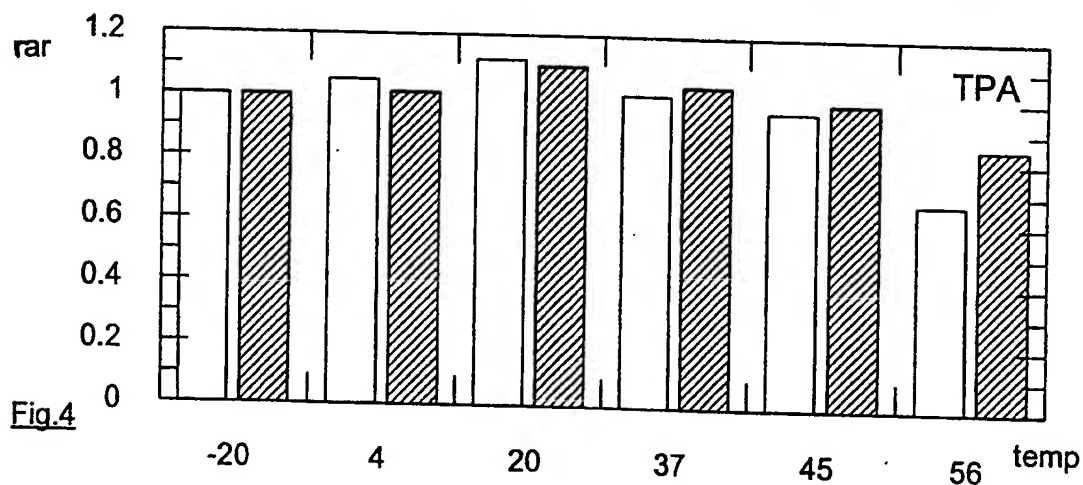
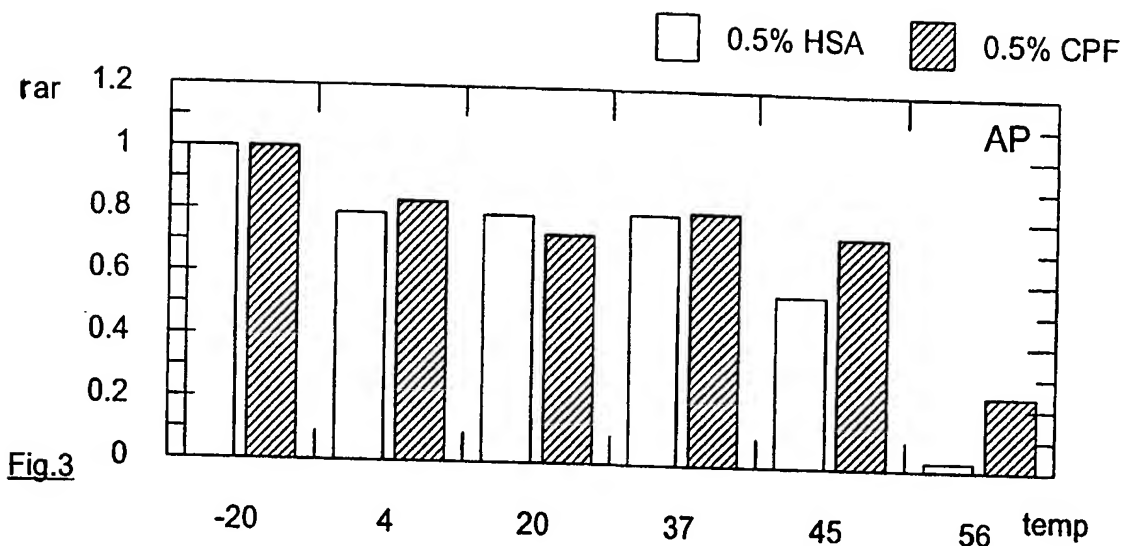


Fig.2

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## INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 96/02453

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C07K14/78 A61K47/42 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K A61K C09H G01N

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## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category * | Citation of document, with indication, where appropriate, of the relevant passages   | Relevant to claim No. |
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| X          | EP,A,0 052 374 (TOPPAN PRINTING CO LTD ;NIPPI INC (JP)) 26 May 1982<br>see page 4, line 15 - page 10, line 26;<br>claims 1,2,6; example 2; tables 1-3<br>---<br>-/-- | 1-4                   |

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| X        | GB,A,2 112 001 (SETON CO) 13 July 1983<br>see page 1, column 2, line 123 - page 2,<br>column 1, line 15; example 1<br>---   | 1                     |
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Information on patent family members

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